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Stable transformation of the moss *Physcomitrella patens*

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Summary. We report the stable transformation of *Physcomitrella patens* to either G418 or hygromycin B resistance following polyethylene glycol-mediated direct DNA uptake by protoplasts. The method described in this paper was used successfully in independent experiments carried out in our two laboratories. Transformation was assessed by the following criteria: selection of antibiotic-resistant plants, mitotic and meiotic stability of phenotypes after removal of selective pressure and stable transmission of the character to the offspring; Southern hybridisation analysis of genomic DNA to show integration of the plasmid DNA; segregation of the resistance gene following crosses with antibiotic-sensitive strains; and finally Southern hybridisation analysis of both resistant and sensitive progeny. In addition to stable transformants, a heterogeneous class of unstable transformants was obtained.

Key words: *Physcomitrella patens* – Transformation

Introduction

We have chosen to use the moss *Physcomitrella patens* as a model for studying the molecular and cellular basis of development. As a model organism, *P. patens* presents the opportunity to study processes of greater morphogenetic complexity than those shown by such algae as *Chlamydomonas rheinhardtii* (Snell 1985), but yet is simpler than higher plants such as *Arabidopsis thaliana* (Meyerowitz 1989). It is easily manipulated in vitro and can be taken through its life-cycle from a single spore or protoplast on a simple mineral medium within 2 to 3 months (Ashton and Cove 1977; Knight et al. 1988). Moreover, as a haploid plant, mutant isolation is facilitated and numerous biochemical and morphological mutants have already been described (Ashton and Cove 1977; Ashton et al. 1988). Genetic complementation analysis of some mutant classes using hybrids produced following protoplast fusion (Grimsley et al. 1977a) has indicated the minimum number of genes involved in certain biochemical pathways or necessary for certain stages of development (Grimsley et al. 1977b; Ashton et al. 1988; Featherstone et al. 1990; C.D. Knight, submitted). In combination with the genetic data, physiological analyses of mutants and the wild-type strain have shown that the plant growth regulators, cytokinin and auxin, are required for cell differentiation both at the single cell level and for the formation of multicellular gametophores (Ashton et al. 1979a, b; Cove and Ashton 1984).

Further similarities are seen with higher plants in that most of the photomorphogenetic processes are mediated by phytochrome (Cove et al. 1978), including protoplast regeneration (Jenkins and Cove 1983a; D. Schaefer, unpublished data), phototropism and polarotropism of protonemata, gametophores (Jenkins and Cove 1983b) and protoplasts (D. Schaefer, unpublished data).

At the molecular level, the sequence of the chlorophyll *a/b*-binding protein gene has been determined and compared with that of higher plants and the alga *Dunaliella salina* (Long et al. 1989). The *P. patens* sequence bears an intron containing enhancer-like elements and 5' sequences that are partially homologous to light-inducible sequences of higher plants. Ribulose bisphosphate carboxylase, small subunit (*rbcS*) genes have also been isolated from a genomic library, using wheat and petunia cDNA probes and are being characterised (D. Schaefer, unpublished data). The restriction pattern and gene order of the chloroplast genome has also been shown to be similar to the consensus land plant genome typified by that of spinach (Calie and Hughes 1987). The continued study of morphogenesis requires analysis at the molecular level and for this purpose a transformation protocol is required. In this paper, we present the first demonstration of stable transformation of *Physcomitrella patens*.

Materials and methods

P. patens culture. Culture conditions for *P. patens* are described by Ashton and Cove (1977) and Knight et al. (1988). Protoplasts were isolated according to Grimsley et al. (1977a) by treating 5- to 7-day-old protonemata with 1%–1.4% (w/v) Driselase (Sigma Chemical Co., Dorset, UK) in 0.44 M mannitol solution for 30–60 min. In Leeds, protoplasts were regenerated according to Knight et al. (1988) in a 2.5-ml layer of medium containing 0.6% (w/v) agar (Oxoid no. 1) and 0.44 M mannitol per 9 cm petri dish, which was separated from a base layer of 1.2% (w/v) agar, 0.33 M mannitol by an 8-cm diameter cellophane disc (W.E. Canning, Bristol, UK). After 4–6 days' incubation in continuous white light (15–20 W/m^2) at 25° C, during which time the regenerating plants reach a stage of 5 to 10 cells, the cellophane overlays were transferred to medium without mannitol and containing the appropriate antibiotic.

In Lausanne, protoplasts were either cultivated in liquid medium for 5 days, and then embedded in a top layer of medium containing 0.6% (w/v) SeaPlaque agarose and regenerated according to Grimsley et al. (1977a) or were directly embedded in a thin layer of 0.6% (w/v) agarose in a 9 cm petri dish and regenerated in beads as described by Shillito et al. (1983). For each treatment, the appropriate antibiotic was applied after 5–7 days. The light regime in Lausanne was 16 h light, 8 h darkness. Cove et al. (1978) showed that continuous light did not adversely affect the morphological pattern of growth and we did not detect any significant difference in transformation frequencies between the two treatments. Visible regenerant plants were further cultivated on selective solid media.

Strains. Table 1 lists the nomenclature and derivation of all *P. patens* strains described in this paper.

Plasmids. Plasmid pLGVneo1103 was obtained from Prof. J Schell (Hain et al. 1985). Plasmids pLGVneo2103 (Hain et al. 1985), pABD1 (Paszkowski et al. 1984), pHP23b (Paszkowski et al. 1988) and pGL2 which consists of a *Bam*HI fragment of the coding sequence of the gene *APH IV* (Gritz and Davies 1983), cloned in the polylinker of pDH51 (Pietrzak et al. 1986), were all kindly provided by Dr. J. Paszkowski (ETH Zurich, Switzerland). pBR322 was obtained from Sigma Chemical Co. Plasmid DNA was isolated and purified using standard procedures (Sambrook et al. 1989). Where applicable, DNA was linearised by restriction endonuclease digestion followed by phenol/chloroform extraction, ethanol precipitation and resuspension in 10 mM TRIS-HCl (pH 7.5)/1 mM EDTA (TE) at 1.0 mg/ml. Restriction enzymes were purchased from Bethesda Research Laboratories and used according to the suppliers' instructions. Calf thymus DNA was sheared to about 5–10 kb and used as carrier DNA.

Table 1. List of strains

Strain	Derivation or reference
Gransden wild-type	Ashton and Cove (1977)
Leman wild-type	Isolated from the banks of Lac Leman, Switzerland, 1982
*pab*A3	Ashton and Cove (1977)
*nic*A4	Ashton and Cove (1977)
*nic*B5/*ylo6*	Ashton and Cove (1977)
c-7	*pab*A3 transformed by pLGVneo1103, selected for G418 resistance at 50 μg/ml
15.03	*pab*A3 transformed by pGL2, selected for hygromycin-resistance at 25 μg/ml
pHP 23 L2	Leman wild-type transformed by pHP23b, selected for G418 resistance at 50 μg/ml

Transformation procedure. The following modifications were made to the method described by Saul et al. (1988). Protoplasts were isolated and washed twice by centrifuging at 800 rpm for 5 min in 0.48 M mannitol. Protoplasts were counted and resuspended at 1.3×10^6/ml in 0.48 M mannitol, 15 mM $MgCl_2$, 0.1% MES-KOH pH 5.6 (MMM solution). Although the inclusion of W5 solution yielded transformants, it resulted in reduced viability and was routinely omitted. Protoplasts were heat shocked at 45° C for 5 min, cooled to 20° C and 300 μl (4×10^5 protoplasts) transferred into 10–14 ml sterile tubes. Thirty microlitres of DNA solution was added to each tube and gently mixed, followed by 300 μl of a solution of 40% (w/v) PEG 4000 (Prolabo) in 0.48 M mannitol containing 0.1 M $Ca(NO_3)_2$, pH 8.0. The PEG (polyethylene glycol) was either autoclaved before dissolving in the sterile mannitol solution or the final solution was sterilised by filtration. The PEG solution was left for 2–3 h before use and made fresh before each experiment.

Thirty microlitres of DNA solution routinely contained 5 μg of plasmid and 25 μg calf thymus carrier DNA (in Lausanne, 3 and 14 μg respectively) although 20–30 μg plasmid DNA only was also effective. The transformation mix was incubated at 20° C for 30 min with occasional gentle mixing. The PEG was diluted from the suspension by progressive step dilution over ca. 30 min with 10 ml of MMM solution. Protoplasts were centrifuged and resuspended in 0.3 ml MMM solution and, as previously described, either placed out in non-selective medium at a density of approximately 10^5 protoplasts per 9 cm petri dish or cultured in 6 ml liquid medium per 6 cm petri dish. Petri dishes were incubated in light (15 W/m^2) for 4–7 days before transfer of the cellophane overlays to medium without mannitol and containing G418 (50 μg/ml) or hygromycin B (25–30 μg/ml). Antibiotic-resistant plants were observed and counted from 10 to 50 days after selection.

Plant DNA analysis. Genomic DNA was isolated from 1–5 g tissue (fresh weight, following drying on a filter by gentle vacuum suction) according to the miniprep procedure described by Dellaporta et al. (1983). This procedure yielded up to 50 μg DNA per gram of plant material. Transformant protonemata were grown on selective medium for at least 7 days, then washed twice in sterile water and filter-dried before freezing in liquid nitrogen. After digestion with restriction enzymes, the DNA was separated by electrophoresis in a 0.7% (w/v) agarose gel, denatured and transferred to nitrocellulose or nylon (Gene Screen Plus or Biodyne) membranes ac-

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cording to standard procedures (Sambrook et al. 1989, or manufacturers' instructions) and hybridised with probes labelled with ^{32}P-dNTP by random hexamer priming (Feinberg and Vogelstein 1983). The genome size of *P. patens* has been estimated to be approximately 7×10^8 bp (N.H. Grimsley and J.-P. Zryd, unpublished data). In general, between 10^6 and 10^7 genomes were loaded per lane with an equivalent number of copies of probe DNA (see legends to the figures).

Crosses. Crosses were made according to Ashton et al. (1988) between an antibiotic-resistant *pab*A3 transformant and the complementary auxotrophic and antibiotic-sensitive strain, *nic*A4. Where transformants had been isolated following treatment of the wild-type strain, crosses were made using the *nicB5/ylo6* strain, in which the *ylo* allele gives a yellow phenotype, to identify products of a cross rather than a self-fertilisation. The strains were co-inoculated in a sterile test tube and grown at 25° C for 3 weeks before transferring to 15° C for 2 weeks and then irrigating with sterile H_2O. In crosses between two auxotrophs, only spore capsules arising as a result of a cross should occur following irrigation with H_2O (Courtice et al. 1978).

Capsules, each containing about 5×10^3 viable spores, appeared after 3–4 weeks and were picked off and stored dry in a sterile Eppendorf tube at 4° C. Individual spore capsules were crushed in sterile H_2O and diluted aliquots spread onto medium supplemented with both *p*-amino benzoic acid and nicotinic acid but without antibiotic. Protonemata from 100 sporelings were inoculated onto complete medium and grown for 10 days before inoculating fragments of each plant onto selective medium supplemented with *p*-amino benzoic acid and nicotinic acid, and non selective medium containing either one or the other vitamin. Plants were grown for 3 weeks before scoring for auxotrophies although segregation of antibiotic resistance was visible after 3 days. Resistant and sensitive progeny were selected and grown for DNA isolation and for Southern hybridisation analysis.

Results

Selection conditions

Protoplasts and protonemata of both wild types and *pab*A3 were tested for their ability to grow on increasing concentrations of kanamycin sulphate, G418 and hygromycin B. On no occasions were surviving colonies detected after 10 days on G418 (10 μg/ml) and hygromycin B (5 μg/ml), whereas reduced growth still occurred on 100 μg/ml of kanamycin sulphate. We therefore decided to select for aminoglycoside phosphotransferase activity using the synthetic aminoglycoside G418 at 50 μg/ml and for hygromycin phosphotransferase activity using hygromycin B at 25–30 μg/ml. An additional advantage to using G418 rather than kanamycin was that G418 has a long life in illuminated culture, thus reducing the need for repeated subculture to maintain selection. Hygromycin degraded over time and cultures were usually subcultured within 2 to 3 weeks to maintain selection.

Table 2. Relative transformation frequencies

Plasmid (promoter)	Plasmid[c] Structure	Selection	Frequency of plants growing (days after transfer to selection)						Total number of stable transformants
							50		
						b			
pLGVneo1103 (*nos*)	s	G418	17	13	37	30	NC	NC	1
pLGVneo2103 (*nos*)	s	G418	12	17	NC	NC	9	13	0
pABD1[d] (CaMV 19S)	s	G418	75	100	24	32	2	2	0
pABD1[d] (CaMV 19S)	l	G418	1	1	1	1	1	1	0
pHP23b (CaMV 35S)	s	G418	84	118	80	112	16	23	2
pHP23b (CaMV 35S)	l	G418	23	25	42	46	24	27	2
pGL2[d] (CaMV 35S)	s	hyg	35	49	13	18	13	18	0
pGL2 (CaMV 35S)	l	hyg	6	8	12	16	12	15	3
No plasmid	-	G418/hyg	0	0	0	0	0	0	0
pBR322 (vector only)	s and l	G418/hyg	0	0	0	0	0	0	0
No PEG	s and l	G418/hyg	0	0	0	0	0	0	0

NC, not counted
a, Frequency per 10^6 viable protoplasts; b, frequency per μg DNA
[c] Supercoiled (s); linear (l)
[d] Data from one experiment only, all other data are the mea of two experiments

Primary selection

The transformation treatment reduces viability to about 10%. The frequencies of antibiotic-resistant plants growing at 10, 27 and 50 days after transfer to selection are presented in Table 2. The total number of stable transformants obtained from both experiments is also shown in Table 2. Controls included treatments where either no DNA or pBR322 DNA (vector-only control) was added and a treatment where the appropriate plasmid but no PEG was added. On no occasions were regenerants detected from these controls using selection levels of 50 μg/ml G418 or 25–30 μg/ml hygromycin B.

It can be seen from Table 2 that the initial transformation frequencies range between 1 and 84 regenerants per 10^6 viable protoplasts or 1 and 118 regenerants per μg DNA. However, the fate of these plants varies. Some regenerants grow strongly on selection and maintain resistance after a period of growth on non-selective media. These represent stable transformants and the frequency of occurrence of this class is low and variable, at best being equal to 10^{-4} viable protoplasts or 1 per μg DNA. Many regenerants do not grow beyond the 100 cells stage (usually reached by 10 days after transfer to selection) and it is likely that these cells have received multiple copies of the resistance gene which are only transiently expressed. A third class of regenerant continues to grow and may be subcultured and maintained for at least 2 years providing that selection is continuous. This class has been obtained frequently in both Leeds and Lausanne and we believe represents unstable transformation which may or may not involve integration (C.D. Knight and D. Schaefer, unpublished data).

It is possible that the structure of the plasmid also influences the transformation frequency and the class of clone recovered. Table 2 shows the number of regenerants obtained for some plasmids either in supercoiled or linear form. In all cases, supercoiled DNA yields a higher initial frequency of antibiotic-resistant regenerants; however, most of these are transient clones.

Mitotic and meiotic stability of stable transformants

The criteria of unrestricted growth on selection and maintenance of resistance after non-selective growth were used as indicators of stable transformation and mitotic stability. Protoplasts were isolated from protonemata of transformants HP 23 L2 and 15.03 which had been grown on non-selective and selective media. The protoplasts were regenerated on media with and without antibiotic and the regeneration frequencies found to be similar, regardless of the initial conditions of growth

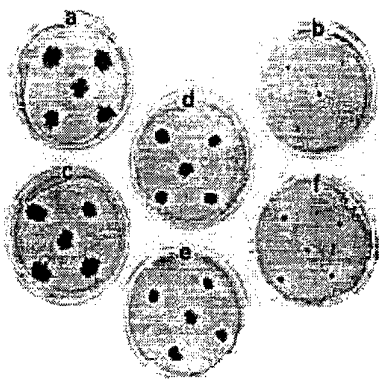

Fig. 1a–f. Each 5 cm plate was inoculated with 5 protonemal inocula of either transformant c-7 (c–f) or the untransformed control *pab*A3 (a, b) and grown for 14 days. At the start of the growth period all plates resembled plate b. Plates a and e do not contain antibiotic. Plates b, d, e and f contain G418 at 50 μg/ml (b, d and e) and 150 μg/ml (f). Plate e was inoculated with a culture grown for 14 days in the absence of selection

(data not shown). A further indication of mitotic stability was noted by the successful fusion of protoplasts and selection of fertile diploid doubly-resistant clones (D. Schaefer, unpublished data). The characteristics of one stable transformant (c-7) are shown in Fig. 1. Transformant c-7 was selected on medium containing G418 at 50 μg/ml but was able to grow at 150 μg/ml G418, albeit with some restriction when compared with the untransformed control under non-selective conditions. The same transformant was tested for its ability to grow on 50 μg/ml after a period of 14 days growth in the absence of selection. Figure 1 shows that this treatment does not affect the growth pattern of transformant c-7.

Hygromycin- and G418-resistant clones were taken through meiosis on selective and non-selective media and spores were tested for their ability to germinate on medium containing antibiotics. A number of different strains stably transformed for either G418 or hygromycin resistance have been shown to transmit resistance to 100% of spores through up to four generations (data not shown).

DNA analysis

Southern hybridisation analysis of a stable transformant revealed the presence of multiple copies of the plasmid DNA integrated at a single site in the nuclear genome. Figure 2 shows the hybridisation pattern of DNA from the G418-resistant transformant c-7, probed with the 2.2 kb *Eco*RI-*Sal*I fragment of the transforming plasmid pLGVneo1103 which contains the *NPT-II* gene (Hain et al. 1985). The quantities of DNA loaded per lane are

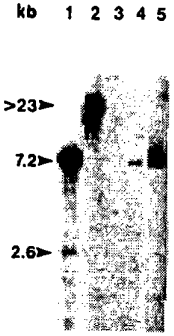

Fig. 2. Southern hybridisation of transformant c-7 probed with the 2.2 kb *Eco*RI-*Sal*I fragment of pLGVneo1103. DNA (3.5 µg) of transformant c-7 was cut with *Sph*I (lane 1) and with *Bgl*II (lane 2); 3.5 µg *Eco*RI-digested DNA of untransformed *pab*A3 was loaded in lane 3 and pLGVneo1103 DNA, linearised with *Eco*RI, was loaded in lanes 4 (35 pg) and 5 (350 pg) (equivalent to 1 and 10 copies per haploid genome, respectively)

described in the figure legend. In comparison with lane 5, which includes the equivalent of 10 linearised plasmid copies, lanes 1 and 2 show that transformant c-7 contains between 20 and 30 copies of the plasmid. Plasmid pLGVneo1103 does not contain a *Bgl*II site and, consequently, digestion wtih this enzyme does not cut the plasmid molecules. The hybridisation signal in lane 2 migrates with the high molecular weight DNA and this is consistent with that predicted for uncut DNA. However, because of the many copies present, this alone is insufficient evidence for integration into the genome. A single *Sph*I site lies within the sequence used as the probe and is situated 1 kb from the *Eco*RI site. When c-7 DNA was digested with *Sph*I an intense band of 7.2 kb was detected indicating that all of the plasmid copies are tandemly arranged. A band of 2.6 kb was also detected and the intensity of this band is the same order as that of the single copy plasmid control in lane 4. We conclude that this band is one of the genomic flanking regions indicating integration into the genome. The second flanking region should also be visible but it is probable that this band is masked by the intense signal at 7.2 kb.

Segregation analysis

In a cross between strains 15.03 (*p*-amino benzoic acid-requiring, hygromycin-resistant) and *nic*A4 (nicotinic acid-requiring, hygromycin-sensitive), spore capsules were obtained after irrigation with H_2O. The progeny of a single capsule were germinated on supplemented media and growth tested for each auxotrophy and hygromycin resistance. The genotypes of 100 progeny are described and analysed statistically in Table 3, and Fig. 3 shows a growth test of a sample of nine progeny. Each allele shows a 1:1 segregation and the *hyg*^{r} allele is unlinked to either the *nic* or *pab* alleles. Similar results were obtained when tissue from the first and second generations of clone HP 23 L2 were each crossed with the strain *nic*B5/*ylo*6 and capsules collected from the yellow colony. Spores were germinated on complete media and then tested for G418 resistance and a yellow phenotype. A 1:1 segregation was recorded for both the G418^r and the *ylo* alleles and neither were linked (data not shown).

Table 3. Analysis of progeny of cross between stable transformant 15.03 (*pab*A3 *hyg*^{r}) and *nic*A4

	yes	5
	no	9
	yes	8
	no	17
	no	21
	yes	12
	no	6
	yes	19
		40:57
		35:62
		44:53[a]
		parentals 47, recombinants 50
		parentals 39, recombinants 58
		parentals 39, recombinants 58

[a] A larger sample of progeny scored only for sensitivity to hygromycin, segregated 101 resistant and 95 sensitive

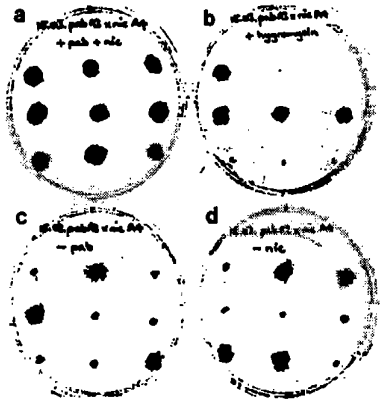

Fig. 3a–d. Nine progeny from a cross between transformant 15.03 × *nic*A4, grown for 3 weeks on medium supplemented with **a** *p*-amino benzoic acid and nicotinic acid; **b** *p*-amino benzoic acid, nicotinic acid and hygromycin; **c** nicotinic acid; and **d** *p*-aminobenzoic acid

DNA analysis of progeny

Genomic DNA was isolated from two hygromycin-resistant and two hygromycin-sensitive progeny of the cross

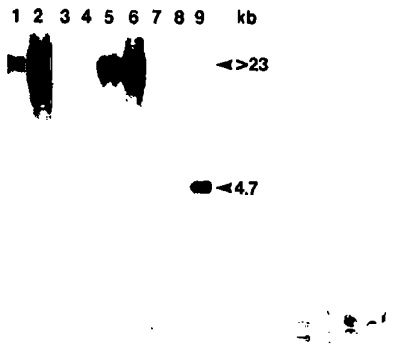

Fig. 4. Southern hybridisation of parent and progeny transformant strains probed with the 1 kb *Bam*HI fragment of plasmid pGL2. Uncut DNA (0.35 µg) of 15.03 was loaded in lane 1 and 3.5 µg *Hin*dIII-cut DNA of the following strains was loaded in each of lanes 2–8: parent transformant 15.03 (lane 2), hygromycin-sensitive 15.03 × *nic*A4 progeny (lanes 3, 4) and hygromycin-resistant progeny (lanes 5, 6). DNA from untransformed *pab*A3 was loaded in lanes 7 and 8. Plasmid pGL2 was linearised by *Hin*dIII digestion and loaded in lanes 8 (24 pg) and 9 (240 pg) (equivalent to 1 and 10 copies per haploid genome, respectively). Longer autoradiographic exposure revealed a 4.7 kb single band in lane 8

described in Table 3 as well as from the parent transformant (15.03). The Southern hybridisation of *Hin*dIII-digested DNA, probed with the 1 kb *Bam*HI fragment of pGL2 which spans the *HPT-II* gene, is shown in Fig. 4. The quantities of DNA loaded per lane are described in the figure legend. Plasmid pGL2 contains a single *Hin*dIII site which lies outside of the sequence used as the probe. Therefore, if all plasmid copies were arranged tandemly, the predicted pattern for this Southern hybridisation would be a single intense band, of 4.7 kb in size, as shown for the control in lane 9 (copy number of 10 per haploid genome). The migration of the hybridisation signal with high molecular weight DNA, as for the uncut DNA treatment in lane 1, can be explained by the fact that since the *Hin*dIII site had been used to linearise the plasmid molecules before transformation, this site must therefore have been destroyed. More importantly, the Southern shows that plasmid molecules are only present in the hygromycin-resistant parent and progeny strains (lanes 2, 5 and 6) and not in the untransformed control and sensitive progeny (lanes 7, 3 and 4).

Discussion

The data presented in this paper demonstrates that *P. patens* is capable of being transformed. Furthermore, G418- and hygromycin-resistant marker genes function in *P. patens* as do the *nos* and cauliflower mosaic virus 19S and 35S promoters, indicating a level of functional comparability between the mosses and higher plants. We conclude that, in stable transformants, plasmid DNA is integrated into the moss genome since these transformants show a Mendelian pattern of inheritance for plasmid-encoded gene function. Conversely, however, the majority of antibiotic-resistant clones obtained by this procedure are unstable and the significance of this class of transformant is currently under investigation.

The relationship between stable and unstable transformants remains unclear but we have some evidence to suggest that some unstable transformants may stabilise with time. However, the most likely way to improve the stable transformation frequency will be to modify the transformation procedure and we are investigating certain possibilities. For example, it has been suggested that integration occurs more frequently when the chromatin structure is relaxed, such as occurs during transcription (Scherdin et al. 1990), following damage induced by X-rays (Koehler et al. 1989) or UV irradiation (Gharti-Chhetri et al. 1990) or in the presence of specific inhibitors of the poly-ADP ribosyl transferase, e.g. benzamide, methylbenzamide or 3-aminobenzamide (Criessen and Shall 1982; Althaus et al. 1982; Gharti-Chhetri et al. 1990). In addition, the inclusion of homologous DNA in the transforming plasmid may promote integration by homologous recombination (Meyer et al. 1989) and we are investigating these treatments.

Although stable transformation must be improved if this technique is to become widely applicable, it should be noted that such low frequencies are not uncommon in higher plant transformation (Gharti-Chhetri et al. 1990). Furthermore, unstable transformation may indeed be advantageous for the delivery of transposons into moss protoplasts since transposition events could be selected for following relaxation of selective pressure, which would eliminate the plasmid vector. This approach is currently being tested as a means of isolating additional morphological mutants by transposon-mediated insertional inactivation (W. Kammerer and D.J. Cove, unpublished data; Cove et al. 1990).

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Note added in proof

The transformation procedure has been improved since the manuscript was submitted. A modified protocol is available upon request.

MGG

Analysis of conserved domains identifies a unique structural feature of a chloroplast heat shock protein

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Summary. A low molecular weight heat shock protein which localizes to chloroplasts has been identified in several plant species. This protein belongs to a eukaryotic superfamily of small HSPs, all of which contain a conserved carboxyl-terminal domain. To investigate further the structure of this HSP, we isolated and sequenced cDNA clones for the chloroplast LMW HSPs from *Petunia hybrida* and *Arabidopsis thaliana*. The cloning of chloroplast HSPs from these two species enabled us to compare the amino acid sequences of this protein from plant species (petunia, *Arabidopsis*, pea, soybean and maize) that represent evolutionarily divergent taxonomic subclasses. Three conserved regions were identified, which are designated as regions I, II and III. Regions I and II are also shared by cytoplasmic LMW HSPs and therefore are likely to have functional roles common to all eukaryotic LMW HSPs. In contrast, consensus region III is not found in other LMW HSPs. Secondary structure analysis predicts that this region forms an amphipathic α-helix with high conservation of methionine residues on the hydrophobic face and 100% conservation of residues on the hydrophilic face. This structure is similar to three helices, termed "methionine bristles", which are found in a methionine-rich domain of a 54 kDa protein component of signal recognition particle (SRP54). The conservation of regions I and II among LMW cytoplasmic and chloroplast HSPs suggests that these HSPs perform related functions in different cellular compartments. However, identification of the methionine bristle domain suggests that chloroplast HSPs also have unique functions or substrates within the special environment of the chloroplast or other plastids.

Key words: Small HSPs – Protein transport – *Arabidopsis* – Methionine bristle – Amphipathic helix

Abbreviations: HS, heat shock; HSP, heat shock protein; LMW, low molecular weight

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Introduction

Like other organisms, plants exposed to elevated temperatures, or heat shock, exhibit stress responses which include the induction of a conserved group of proteins, the heat shock proteins (Lindquist and Craig 1988). The most abundant HSPs synthesized by most higher plants are a group of small proteins of between 15 and 30 kDa. These low molecular weight HSPs are found in the cytoplasm, as seen in other eukaryotes, but in addition a homologous LMW HSP has also been identified which localizes to chloroplasts (Kloppstech et al. 1985; Vierling et al. 1986). The chloroplast-localized LMW HSP has been identified in pea (*Pisum sativum*), soybean (*Glycine max*), maize (*Zea mays*), bean (*Phaseolus vulgaris*), *Arabidopsis thaliana* and *Chlamydomonas reinhardtii* (Vierling et al. 1986, 1989; Nieto-Sotolo et al. 1990; Kloppstech et al. 1985; Süss and Yordanov 1986). It is a nuclear-encoded protein which is synthesized as a precursor in the cytoplasm and post-translationally transported into chloroplasts. The amino acid sequences of LMW chloroplast HSPs have been derived from the DNA sequence of cDNA clones from pea, soybean and maize and revealed that chloroplast HSPs belong to a superfamily of small HSPs, all of which contain a conserved carboxyl-terminal heat shock domain (Vierling et al. 1988; Nieto-Sotolo et al. 1990).

To investigate further the structure of chloroplast LMW HSPs, we isolated and sequenced cDNA clones for the chloroplast LMW HSPs from two dicotyledonous plants: petunia (*Petunia hybrida*) and *Arabidopsis* (*A. thaliana*). These species represent taxonomic subclasses which are widely diverged relative to those in which chloroplast HSPs have been previously characterized. A comparison of the amino acid sequences of the LMW chloroplast HSPs from these two species with those of pea, soybean and maize led to the identification of three consensus regions. In addition to the carboxyl-terminal heat shock domain and another domain which are shared by cytoplasmic LMW HSPs, chloroplast